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Functional analysis of recombinant codon-optimized bovine neutrophil β-defensin

Shahrzad Aghaei [a], Behnaz Saffar [a,b,*], Kamran Ghaedi [c], Mohsen Mobini Dehkordi [a]

[a] Department of Genetics, Faculty of Sciences, Shahrekord University, Shahrekord, Iran
[b] Biotechnology Research Institute, Shahrekord University, Shahrekord, Iran
[c] Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran

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ABSTRACT

Defensins are cationic antimicrobial peptides with a broad range of activities against bacteria and fungi. In the present study, the entire coding sequence of codon-optimized Bovine Neutrophil β-Defensin 2 (BNBD2) was designed and placed upstream of *Trx* coding sequence into the pET-48b (+) vector. Furthermore, the codon-optimized pelB signal sequences were also added to the upstream of BNBD2 for periplasmic localization. The periplasmic sorting of recombinant β-Defensin 2 was evaluated by osmotic shock and SDS–PAGE on the released proteins. Moreover, the expression of BNBD2-Trx fusion protein was confirmed by the Western blotting technique. Next, the purification of recombinant protein was achieved by Ni^{++} affinity chromatography. BNBD2 was also separated from Trx by chemical cleavage with formic acid. Finally, both of the antibacterial and antifungal activities of the purified protein were examined. Overall, the results indicated successful periplasmic production of BNBD2 protein, which showed antifungal activity against some of *Aspergillus* species as well as the antibacterial activity, expressed as successfully suppressed growth of *Escherichia coli* and *Staphylococcus aureus*.

Introduction

Antimicrobial peptides, (AMPs), display antimicrobial activity against gram-negative and -positive bacteria, yeasts, and fungi [1,2]. One of the largest families of AMPs is the defensins which are found in vertebrates, invertebrates, and plants. Defensins are cationic peptides with 18–45 amino acid residues. They have a molecular weight of 2–6 kDa with a framework of 6 Cysteine residues (3 disulfide bonds) [3]. Based on the relative positions of disulfide bonds, defensins are classified as α, β or θ-defensins [4]. They exhibit various antimicrobial functions. β-Defensins are able to interact with the charge-negative cellular membranes of bacteria with high affinity. Due to the changes in the membrane structure and electric potential, these peptides can be inserted into the phospholipid layers of the membranes. Hence, they cause membrane depolarization and cell lysis. Antimicrobial effects of β-defensins

* Corresponding author. Tel.: +98 3832324401-5; fax: +98 3832324419.
E-mail address: saffar_b@sci.sku.ac.ir (B. Saffar).
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depend on the hydrophobicity and distribution of positive charge amino acids [5]. In domestic cattle, 13 types of β-defensins with conserved sequences were found in blood neutrophil granulocytes [6]. Bovine neutrophil β-defensins (BNBD1–13) are arginine-rich peptides and include 38–42 amino acids with antibacterial activity [7,8]. Therefore, studies on BNBDs could develop a new type of antibiotics in the treatment of mastitis in dairy cows. The N-terminal post-translational modification has been detected in some of BNBDs; however, the main modification of BNBD2 is the formation of disulfide linkages [6]. To the best of our knowledge, no study has been done on the production of recombinant BNBD2 so far. Therefore, BNBD2 can also be a good candidate for the production of recombinant antimicrobial peptides in *Escherichia coli*.

Cytoplasmic production of recombinant proteins in *E. coli* is accompanied by cellular disruption and contamination of the target proteins with other cytoplasmic components [9]. However, targeting recombinant protein in the bacterial periplasmic space has the advantage of having less onerous purification and proteolysis, authentic N-terminus and proper folding, and suitable biological activity [10,11]. In addition, the periplasm has a more oxidizing environment than the cytoplasm, and favors the formation of the disulfide bonds, which is important for the activity of respective proteins [12]. Typically, a signal peptide such as pelB, derived from pectate lyase B of *Erwinia carotovora*, is introduced at the N-terminus of recombinant proteins to help drive them into the periplasmic space [13,14]. Moreover, codon optimization of recombinant proteins or removal of rare codons can increase the efficiency of protein production in *E. coli* [15,16]. In this study, the production of a codon-optimized pelB-BNBD2 with an appropriate direction into the periplasmic space was found. Notably, this protein exhibited antibacterial as well as antifungal activities.

Material and methods

Construction of the recombinant vector

E. coli, BL21 (DE3) F-ompT hsdsB ($r^{-}Bm^{-}$) gal dcm, and pET48b (+) were used as the host and vector, respectively. Coding sequences (CDS) of the bovine *BNBD2* gene (Accession number, P46160), were obtained via University of California Santa Cruz (UCSC) data bank. Specific sequences, encoding PelB signal peptide and cleavage site of formic acid (Asp-Pro), were placed at the 5′ and 3′-terminal ends of *BNBD2* CDS, respectively (Fig. 1). Two web servers, optimizer (http://www.genoms.uvr.es), and *E. coli* rare codon analyzer 2 (http://www.faculty.ucr.edu), were performed on *BNBD2* CDS. Meanwhile, Codon Adaptation Index (CAI) was used to evaluate the designed sequences to the Gene script web site (http://www.gene script.com) (Table 1). The optimized sequences (250 bp) of *pelB-BNBD2* were ordered from the NedayeFan Company (Tehran, Iran). Synthesized DNA fragments which had been inserted into the pGH vector were digested with *Xba*I/*Nde*I. Subsequently, *Xba*I-*pelB-BNBD2-Nde*I was inserted into the same place in pET48b (+). The ligation was achieved using T4 ligase enzyme (TaKaRa, Japan). And finally, the DNA manipulations were carried out according to the standard protocols [17].

Protein expression

A single colony of recombinant bacteria transformed with pET48b (+)-*BNBD2* was cultured overnight in 5 mL of LB broth medium which contained 30 μg Kanamycin/mL at 37 °C. The overnight culture was transferred to 50 mL of LB broth medium. Upon reaching the optical density (OD_{600}) of 0.4–0.6, the protein synthesis was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). The optical density was measured by the spectrophotometer (Shimadzu, Japan). Afterward, the temperature of the incubator was set to 30 °C. After another 2–6 h incubation period, the cell pellets were harvested and re-suspended in TES buffer (Tris 20 mM-EDTA 1 mM, pH: 8, 20% sucrose). The cells were incubated on ice for 30 min, centrifuged and resuspended in 50 mM of ice-cold $MgSO_4$, and incubated again on ice for another 30 min. The periplasmic proteins were collected via centrifugation at 12,000*g* (4 °C) [18]. Further, the supernatants were concentrated by Trichloroacetic acid (TCA). Later, equal amounts of periplasmic protein and the pellet as cytoplasmic protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). In addition, the total protein was obtained by the sonication method with TE buffer (10 mM Tris, 1 mM EDTA, pH: 7.8) for 30 s and at 50/60*Hz*.

Western Blotting Analysis

A solubilized protein fraction of each sample was subjected to SDS–PAGE and transferred to a polyvinylidene difluoride

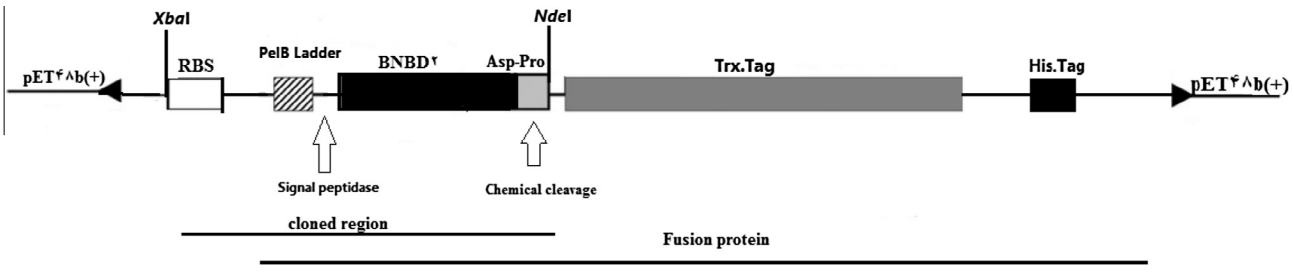

Fig. 1 Schematic representation for a part of the expression vector pET48b (+) - BNBD2. A codon optimized DNA fragment encoding pelB-*BNBD2* was inserted upstream of Thioredoxin (Trx) encoding sequences in pET48b (+) vector. BNBD2 was expressed as a fusion protein with Trx. As depicted, (Asp-Pro) was designed to facilitate chemical cleavage for release of recombinant BNBD2 from the fusion protein.

Table 1 Sequence comparison between optimized and native codons of the pelB-*BNBD2*-(Asp-Pro) sequence.

N	ATG	AAA	TAC	CTG	CTG	CCG	ACC	GCT	GCT	GGT	CTG	CTG	CTC	CTC	GCT	GCC	CAG
O	ATG	AAA	TAT	CTG	CTG	CCG	ACC	GCC	GCG	GGT	CTG	CTG	CTG	CTG	GCT	GCA	CAA
a.a	M	K	Y	L	L	P	T	A	A	G	L	L	L	L	A	A	Q
N	CCG	GCG	ATG	GCC	ATG	GTG	CGC	AAC	CAT	GTG	ACC	TGC	CGC	ATT	CGC	GGC	TTT
O	CCG	GCG	ATG	GCT	ATG	GTC	CGT	AAC	CAT	GTA	ACC	TGC	CGT	ATT	CGT	GGT	TTC
a.a	P	A	M	A	M	V	R	N	H	V	T	C	R	I	N	R	G	F
N	TGC	GTG	CCG	ATT	CGC	TGC	CCG	GGC	CGC	ACC	CGC	CAG	ATT	GGC	ACC	TGC	TTT	GGC
O	TGT	GTA	CCT	ATT	CGT	TGC	CCT	GGT	CGC	ACT	CGT	CAG	ATC	GGT	ACG	TGT	TTT	GGC
a.a	C	V	P	I	R	C	P	G	R	T	R	Q	I	G	T	C	F	G
N	CCG	CGC	ATT	AAA	TGC	TGC	CGC	AGC	TGG	CGA	TGA	CGA	TCC	TAT				
O	CCG	CGT	ATT	AAA	TGC	TGC	CGT	AGT	TGG	CGA	TGA	CGA	TCC	TAT				
a.a	P	R	I	K	C	C	R	S	W	D	D	D	P	I				

The bold ATG codon shows start codon of BNBD2 sequence. The bold nucleotides in native codons were changed as described in Materials and methods. Underline codons are rare codons which were optimized. N and O and a.a. are referred as native sequence, optimized sequence, and amino acid residue respectively.

(PVDF; Bio-Rad, USA) membrane which had been activated by soaking it in methanol for 15 s. After blocking the membrane with TBST-5% (w/v) skim milk for 2 h at room temperature, the membrane was incubated with a dilution of mouse anti-(6×His) peroxidase antibody (1:2000, Sigma, USA). The HRP-conjugated IgG bound to each protein band and was visualized by adding the Tetramethylbenzidine (TMB) substrate [17].

Purification of Recombinant Protein

The purification procedure was carried out using Ni^{++} affinity chromatography (His-Bind Quick 300 Cartridges, 70155–3, Novagen) as described by Swartz [19]. The column was washed with Wash Buffer (100 mM Tris base, 500 mM NaCl, and 10 mM imidazole, pH: 8) and double sterile H_2O, respectively, and then was charged with 100 mM NiSO4. A 10 mL volume of periplasmic protein sample (obtained from 350 mL of recombinant bacterial cultures) was passed through a column, and the column was washed with the binding buffer (100 mM Tris base, 500 mM NaCl, and 20 mM imidazole, pH:8) to remove non-specific bound proteins. Finally, the recombinant fusion protein was eluted by adding Elution buffer (100 mM Tris base, 500 mM NaCl, and 500 mM imidazole, pH: 8). The eluted fractions were collected, and the protein concentrations were determined by the Bradford assay [20]. The purified protein was concentrated by a 10 kDa cutoff dialysis membrane for removing small peptides. The Fusion proteins were then digested by treating them with formic acid at final concentration of 50% (v/v) for 24 h in 45 °C according to the method described by Li et al., with some modifications [21]. The digested products were freeze-dried for 24 h to remove the formic acid. Lastly, Tricine–SDS–PAGE 16% was employed to detect the purified BNBD2. The mature BNBD2 was again purified with a Centricon tube (Sartorius Stedim biotech GmbH) at 4000 g centrifugation and 4 °C for 40 min.

Antifungal and antibacterial activities assays

The antifungal effects of the purified BNBD2 protein were tested against *Aspergillus flavus (PTCC 5004)* and *Aspergillus parasiticus (PTCC 5018)*. These strains were purchased as freeze-dried stock (Persian Type Culture Collection, Tehran, Iran). The regeneration of these stocks was performed by adding a sterile solution of Tryptic Soy Broth (TSB) (Merck & Co., Inc., USA). This suspension was spread on solid Sabouraud Dextrose Agar (SDA) media and was incubated at 37 °C for mycelium growth of fungi. The $10E^4$ spores of fungi were collected aseptically from 10 day-old cultures after neobar lam calculation. The spore suspensions were later plated on Mueller-Hinton (M-H) agar, supplemented with 2% glucose and methylene blue [22]. The fungi were allowed to grow for two days at 37 °C in the central zone of each plate. Standard paper disks containing different concentrations of purified BNBD2 (0.05, 0.1 and 0.2 mg/mL) were placed on these M-H plates. The fusion type of BNBD2-Trx was used as the negative control, while a kresoxim-methyl antifungal compound was used in two concentrations of 50 and 100 ppm as the positive control. The plates were incubated at 37 °C for 48 h. The antibacterial effects of BNBD2 were assessed by suppressing the growth of *Staphylococcus aureus* (ATCC25923)

and *E. coli* (ATCC35218) as typical gram positive and negative bacteria via a gel diffusion method. The bacterial cells were grown for lawn seeding in TSB media. Then, the bacterial inoculates were adjusted to 0.5 McFarland standards and were completely spread on solid M-H agar plates by surface culture method using sterile swabs. The wells were created on the surface of the agar plate in the presence of 0.05, 0.1, and 0.2 mg/mL of BNBD2 and under sterile conditions. Similar to the previous experiments, BNBD2-Trx was used as negative control. The plates were then incubated at 37 °C overnight.

All of the antifungal and antibacterial activity assays and the Western blotting analysis were carried out in triplicate.

Results

Codon optimization and cloning

In order to produce bovine BNBD2 in *E. coli* appropriately, the codon optimization was performed. The gene script web server was implemented so as to calculate Codon Adaptation Index (CAI) of the native bovine BNBD2 CDS. The data indicated that the CAI was 0.61. Thus, the codon optimization was performed and CAI was measured again to be 0.83, which was an appropriate index of expression. After obtaining BNBD2 CDS, it was subcloned in pET48b (+), resulting in the production of pET48b (+)-*BNBD2* (as described in detail in the Materials and methods section). Proper construction of pET48b (+)-*BNBD2* was confirmed by digestion of the recombinant vector with *Xba*I/*Nde*I restriction enzymes, yielding two fragments (250 bp and 5560 bp) (Fig. 2). DNA sequencing was performed to ensure that no mutations were present in the sequence of BNBD2 CDS (Data not shown).

Expression and purification of recombinant protein and Western blotting technique

The recombinant vector was transformed into the *E. coli* cells to produce the recombinant protein. Afterward, the protein production was carried out (as described in the Materials and methods section). SDS–PAGE gel on proteins released by osmotic shock indicated a band with an approximate size of 24 kDa, which was related to the production of BNBD2 fused with Trx-6×His. A similar band was also detected in the bacterial lysate cells (Fig. 3A). Furthermore, the purification of recombinant BNBD2-Trx-6×His was performed, and the purified fusion protein was again subjected to the SDS–PAGE, (Fig. 3B). To ensure the fact that the detected band was the respective band of BNBD2-Trx-6×His, the Western blotting was carried out by implementing an antibody against 6×His tags (Fig. 3C). The concentration of proteins was estimated to be about 4.2 mg/mL. The yield of purified recombinant protein was approximately 12 mg/L of culture media. Finally, formic acid was used to separate BNBD2 from the carrier protein (Trx-6×His) (Fig. 3D).

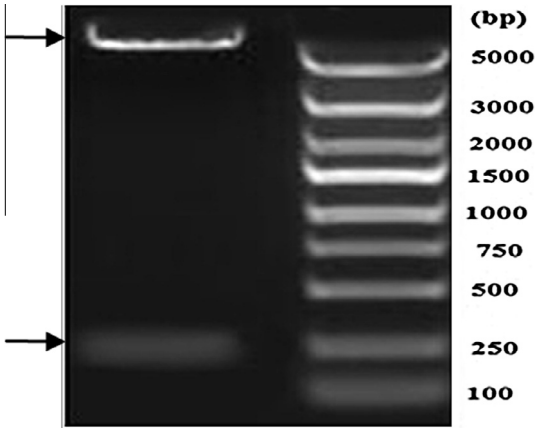

Fig. 2 Agarose gel electrophoresis of double digestion of plasmid pET48b (+)-BNBD2 using *Xba*I/*Nde*I restriction enzymes. The arrows show desired 250 bp and 5560 bp band respective to the BNBD2 CDS and the backbone of plasmid. Marker (DL 5000 DNA Marker, China).

Antifungal and antibacterial assays

The antifungal effects of BNBD2 peptide were examined by the potency of recombinant peptides to inhibit the growth of two different fungi species. The data indicated a clear inhibition zone of recombinant peptides when subjected to the plate of fungi (Fig. 4).

Additionally, the antibacterial activity of BNBD2 was tested by subjecting the recombinant peptide to the growth culture of *E. coli* and *S. aureus* (as described in the Materials and methods section). Recombinant purified BNBD2 inhibited the growth of bacteria compared to the BNBD2-Trx-6×His (Fig. 5). These results showed that BNBD2 was functional and had a strong potential against fungi and bacterial growth compared to the negative control.

Discussion

Most defensins have antibacterial and antifungal activities [7]. As a result of the high activity of defensins *in vitro*, it appears that the production of defensin would hold promise in applications for antibacterial and antifungal treatments in the future. *E. coli* cells are recognized as suitable hosts for recombinant protein production due to their rapid growth, simple genetic background, feasibility for high scale production, and cost-effectiveness [23].

One of the most important issues which should be considered is the codon optimization of proteins in heterologous host cells. Codon optimization could improve the expression of eukaryotic proteins in *E. coli* [15]. Hence, the expression of recombinant human B defensin-2 (hBD2) was reported to increase 9 fold after the codon optimization [24]. Tiwari et al. showed that codon optimization and removal of rare codons could increase periplasmic expression of proteins more than 100 fold [25]. In the present study, BNBD2 is an arginine rich peptide with 8 arginine residues. Therefore, rare codons of arginine (CGC) were subjected for optimization. In the previous works on BNBD2, the native sequences of the *pelB-BNBD2* gene were cloned into pET48b (+) in an approach similar to that of this study. However, the amount of recombinant protein was not significant, and the expression was not satisfactory (unpublished data). Hence, in this study, the codon optimization was done for the appropriate production of BNBD2. The pET48b (+) vector containing Trx-tag (as a

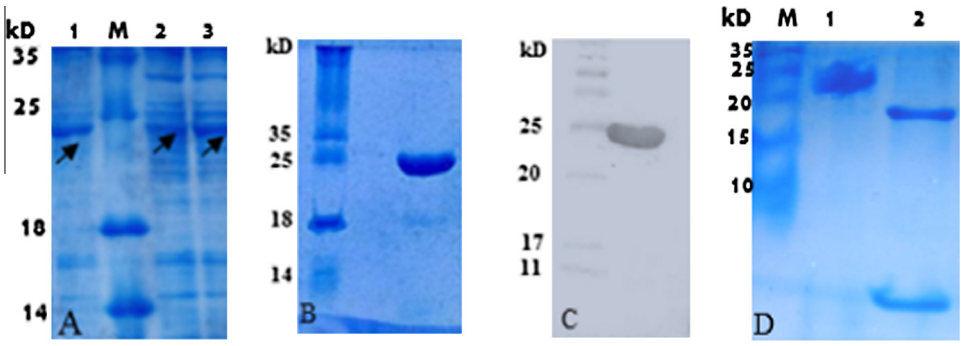

Fig. 3 Analysis of expression, purification, and cleavage of BNBD2-Trx-6×His recombinant protein. (A) SDS–PAGE analysis of protein expression of *E. coli* BL21 (DE3) transformed with pET48b (+)-BNBD2-Trx-6×His, 2 h after induction with 1 mM IPTG. Lane 1, the periplasmic proteins after osmotic shock. Lane M, Marker SMO431. Lane 2, the pellet left as cytoplasmic protein. Lane 3, the total protein pattern. The desired bands are marked with arrows. (B) BNBD2-Trx-6×His recombinant protein purified by Ni^{++} affinity chromatography. (C) Analysis of Western blotting of BNBD2-Trx-6×His recombinant protein that is carried out by implementing an antibody against 6×His tag. (D) Peptide release followed by Tris-Tricine SDS–PAGE (16%). M, Protein marker. Lane 1, the recombinant protein BNBD2-Trx-6×His before cleavage. Lane 2, the recombinant protein, BNBD2-Trx-6×His after cleavage with formic acid. Approximately a 3–8 kDa band as BNBD2 peptide after cleavage is shown.

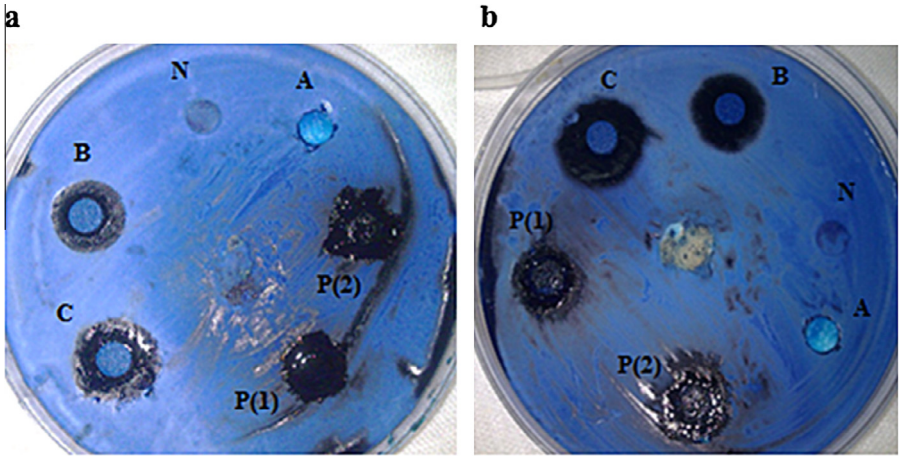

Fig. 4 Analysis of the antifungal effects of BNBD2 by the disk diffusion method on (a) *Aspergillus flavus (PTCC 5004)* and (b) *Aspergillus parasiticus* (PTCC5018). N: Negative control (recombinant protein BNBD2-Trx), P (1): Positive control: (Kresoxim-methyl 50 ppm), P (2): Positive control (Kresoxim-methyl 100 ppm). A, B and C: 0.05 mg/mL, 0.1 mg/mL and 0.2 mg/mL recombinant purified BNBD2. The results show inhibition zones of purified BNBD2 peptides.

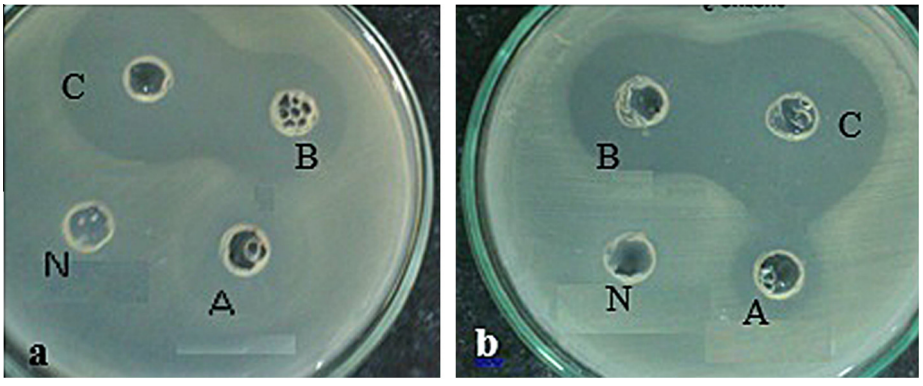

Fig. 5 Antimicrobial activities of BNBD2 assayed by the agar gel diffusion method. Inoculates of *E. coli* (ATCC 35218) (a) and *S. aureus* (ATCC 25923) (b) were spread on solid agar M-H medium plate. N: Negative control is fusion protein BNBD2-Trx. A, B and C: 0.05 mg/mL, 0.1 mg/mL and 0.2 mg/mL recombinant purified BNBD2 protein.

carrier protein) was used as an expression vector. Due to the small size of BNBD2 (3–8 kDa), Trx-6×His facilitated not only the identification of recombinant BNBD2 on SDS–PAGE but also the solubilization of BNBD2. It also inhibited inclusion body (Ib) formation of the desired recombinant protein. However, the presence of the carrier protein significantly suppressed the antibacterial and antifungal activities of recombinant BNBD2 in *E. coli*. This showed that the presence of this carrier protein in *E. coli* cells was necessary in preventing the toxic effects of BNBD2 on bacterial host cells. Formic acid was used to release BNBD2 from the carrier protein. One or two aspartate residues from the formic acid cleavage site may remain at the C-terminus of the recombinant BNBD2. Li et al. stated that although the Asp-x bond is most readily cleaved by formic acid, the x-Asp may also be cleaved. Therefore, the resulting peptides may keep at least one aspartyl residue [26]. Due to the negative charge of Asp, adding one or two Asp at C terminal of BNBD2 may have a negative effect on protein activity; nevertheless, it has the lowest unfavorable effect in comparison with other reagents such as enterokinase or thrombin.

The protein pattern obtained from the total, periplasmic, and cytoplasmic proteins showed that despite the fact that the amount of recombinant protein remains in the cytoplasm, a major part of the expressed recombinant BNBD2 was directed to periplasmic space. This issue can be accounted for by the solubility of the recombinant protein which caused efficient secretion of BNBD2 to periplasmic space. While the majority of antimicrobial peptides have cysteine residues and disulfide bonds, periplasm with a more oxidative space than the cytoplasm provides the formation of disulfide bonds, true folding, and better activity of the protein. Zhao et al., reported that tertiary structure is necessary for antimicrobial activity of defensins and increases resistance to bacterial proteases [27]. BNBD2 contains 3 disulfide bonds which play an important role in the formation of the 3D structure. Therefore, it is assumed that periplasmic space is a more favorable environment for the expression of BNBD2. Moreover, there is less proteolysis and even less onerous purification in this space [28]. Despite the previous unsuccessful reports [29] on periplasmic expression of thionine (as an antimicrobial peptide), this study successfully showed the periplasmic expression of an antimicrobial peptide (BNBD2) for the first time. Based on the research findings of this study, the pelB signal peptide was suitable for periplasmic expression of this peptide. The periplasmic space could be a privileged target for purifying the target protein.

Wu et al. were able to express BNBD12 protein by pET32a (+) in *E. coli*. They concluded that BNBD12 had antimicrobial activity in *E. coli* and *S. aureus* and can control mastitis [7]. Added to that, Simon et al., reported potent antifungal properties of human peptide homolog with drosomycin, drosomycin-like defensin (DLD) in *Aspergillus* (*A. fumigates*, *A. nidulans*, *A. ustus*) and *Fusarium* species [30]. Kazakos et al., demonstrated the potent antifungal properties of the human beta defensins (hBD1, hBD2, and hBD3) against representative clinical isolates of filamentous fungi; including *Aspergillus fumigatus*, *Aspergillus niger*, *A. nidulans*, and *Aspergillus terreus* [31]. To investigate the activity of the protein, the antimicrobial activities of BNBD2 were analyzed on selected standard model bacteria, i.e. *E. coli* as a gram negative bacterium, *S. aureus* as a gram positive bacterium, and two major aflatoxigenic fungi; named *A. flavus* and *A. parasiticus* (which cause liver cancer in humans) [32]. The findings showed that BNBD2 inhibited the growth of both gram negative and -positive bacteria and fungi. This semi-quantitative study continues to search for protein activity, and more characterization of BNBD2 needs to be discovered.

Finally, in the future, BNBD2 can be used to control mastitis in dairy cows. Despite the existence of antifungal and antimicrobial properties of recombinant BNBD2, further extensive research on the conformation of this type of BNBD2 is required to ensure that proper disulfide bonds are produced.

Conclusions

A codon optimized pelB-BNBD2 gene for the periplasmic expression was constructed by the pET48b (+) vector. Periplasmic expression and purification were successful. Antimicrobial quantitative study of recombinant BNBD2 on *E. coli*, *S. aureus*, and two *Aspergillus sp*. showed growth inhibition. Despite the presence of an activity for recombinant BNBD2, more efforts should be carried out on characterization of recombinant BNBD2 to obtain a realistic view of valuable data on the expression and antimicrobial properties. In particular, this activity should be quantified compared to the conventional antibiotics. Taken together, such studies on BNBD2 could develop a new generation of antibiotics that might be effective for treatment of some dairy diseases, such as mastitis in near future.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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